DESCRIPTION

NEMATICIDAL PROTEINS

Cross-Reference to Related Applications

[0001] This is a division of co-pending application Serial No. **09/738,363** *(filed December 15, 2000)* which is a division of application Serial No. **09/076,137** *(filed on May 12, 1998, which issued as U.S. Patent No. 6,166,195 on December 26, 2000)* which is a division of application Serial No. **08/316,301** *(filed on September 30, 1994, which issued as U.S. Patent No. 5,753,492 on May 19, 1998)* which is a division of application Serial No. **07/871,510** *(filed on April 23, 1992, now abandoned)* which is a continuation-in-part of application Serial No. **07/693,018** *(filed on May 3, 1991, now abandoned)* and a continuation-in-part of application Serial No. **07/830,050** *(filed on January 31, 1992, now abandoned)*. Serial No. **07/693,018** was a continuation-in-part of Serial No. **07/565,544** *(filed on August 10, 1990, now abandoned)* which is a continuation-in-part of application Serial No. **07/084,653** *(filed on August 12, 1987, now U.S. Patent No. 4,948,734)*. The subject application is also a continuation-in-part of application Serial No. **07/675,772** *(filed March 27, 1991, now U.S. Patent No. 5,262,399)* which is a continuation-in-part of Serial No. **07/565,544** and a continuation-in-part of Serial No. **07/557,246** *(filed on July 24, 1990, now U.S. Patent No. 5,281,530)*. Serial No. **07/557,246** is a continuation-in-part of Serial No. **07/535,810** *(filed June 11, 1990, now abandoned)* which is a continuation-in-part of Serial No. **07/084,653**.

Background of the Invention

[0002] Regular use of chemicals to control unwanted organisms can select for chemical resistant strains. This has occurred in many species of economically important insects and has also occurred in nematodes of sheep, goats, and horses. The development of chemical resistance necessitates a continuing search for new control agents having different modes of action.

[0003] In recent times, the accepted methodology for control of nematodes has centered around the drug benzimidazole and its congeners. The use of these drugs on a wide scale has led to many

instances of resistance among nematode populations (Prichard, R.K. et al. [1980] "The problem of anthelmintic resistance in nematodes," Austr. Vet. J. 56:239-251; Coles, G.C. [1986] "Anthelmintic resistance in sheep," In *Veterinary Clinics of North America: Food Animal Practice*, Vol 2:423-432 [Herd, R.P., eds.] W.B. Saunders, New York). There are more than 100,000 described species of nematodes.

[0004] The bacterium *Bacillus thuringiensis* (*B.t.*) produces a δ-endotoxin polypeptide that has been shown to have activity against a rapidly growing number of insect species. The earlier observations of toxicity only against lepidopteran insects have been expanded with descriptions of *B.t.* isolates with toxicity to dipteran and coleopteran insects. These toxins are deposited as crystalline inclusions within the organism. Many strains of *B.t.* produce crystalline inclusions with no demonstrated toxicity to any insect tested.

[0005] A small number of research articles have been published about the effects of delta endotoxins from *B. thuringiensis* species on the viability of nematode eggs. Bottjer, Bone and Gill (Experimental Parasitology 60:239-244, 1985) have reported that *B.t. kurstaki* and *B.t. israelensis* were toxic in vitro to eggs of the nematode *Trichostrongylus colubriformis*. In addition, 28 other *B.t.* strains were tested with widely variable toxicities. The most potent had LD_{50} values in the nanogram range. Ignoffo and Dropkin (Ignoffo, C.M. and Dropkin, V.H. [1977] J. Kans. Entomol. Soc. 50:394-398) have reported that the thermostable toxin from *Bacillus thuringiensis* (beta exotoxin) was active against a free-living nematode, *Panagrellus redivivus* (Goodey); a plant-parasitic nematode, *Meloidogyne incognita* (Chitwood); and a fungus-feeding nematode, *Aphelenchus avena* (Bastien). Beta exotoxin is a generalized cytotoxic agent with little or no specificity. Also, H. Ciordia and W.E. Bizzell (Jour. of Parasitology 47:41 [abstract] 1961) gave a preliminary report on the effects of *B. thuringiensis* on some cattle nematodes.

[0006] At the present time there is a need to have more effective means to control the many nematodes that cause considerable damage to susceptible hosts. Advantageously, such effective means would employ biological agents.

Brief Summary of the Invention

[0007] The subject invention concerns novel toxins active against nematodes. A further aspect of the invention concerns genes coding for nematicidal toxins. The subject invention provides the

person skilled in this art with a vast array of nematicidal toxins, methods for using these toxins, and genes that code for the toxins.

[0008] One aspect of the invention is the discovery of two generalized chemical formulae common to a wide range of nematicidal toxins. These formulae can be used by those skilled in this art to obtain and identify a wide variety of toxins having the desired nematicidal activity. The subject invention concerns other teachings which enable the skilled practitioner to identify and isolate nematode active toxins and the genes which code therefor. For example, characteristic features of nematode-active toxin crystals are disclosed herein. Furthermore, characteristic levels of amino acid homology can be used to characterize the toxins of the subject invention. Yet another characterizing feature pertains to immunoreactivity with certain antibodies. Also, nucleotide probes specific for genes encoding toxins with nematicidal activity are described.

[0009] In addition to the teachings of the subject invention which define groups of *B.t.* toxins with advantageous nematicidal activity, a further aspect of the subject invention is the provision of specific nematicidal toxins and the nucleotide sequences which code for these toxins.

[0010] One aspect of the of the subject invention is the discovery of two groups of *B.t.*-derived nematode-active toxins. One group (CryV) is exemplified by the gene expression products of PS17, PS33F2 and PS63B, while the other group (CryVI) is exemplified by the gene expression products of PS52A1 and PS69D1. The organization of the toxins within each of the two groups can be accomplished by sequence-specific motifs, overall sequence similarity, immunoreactivity, and ability to hybridize with specific probes.

[0011] The genes or gene fragments of the invention encode *Bacillus thuringiensis* δ-endotoxins which have nematicidal activity. The genes or gene fragments can be transferred to suitable hosts via a recombinant DNA vector.

Brief Description of the Sequences

[0012] **SEQ ID NO. 1** discloses the DNA of 17a.

[0013] **SEQ ID NO. 2** discloses the amino acid sequence of the toxin encoded by 17a.

[0014] **SEQ ID NO. 3** discloses the DNA of 17b.

[0015] **SEQ ID NO. 4** discloses the amino acid sequence of the toxin encoded by 17b.

[0016] **SEQ ID NO. 5** is the nucleotide sequence of a gene from 33F2.

[0017] **SEQ ID NO. 6** is the amino acid sequence of the protein expressed by the gene from 33F2.

[0018] **SEQ ID NO. 7** is the nucleotide sequence of a gene from 52A1.

[0019] **SEQ ID NO. 8** is the amino acid sequence of the protein expressed by the gene from 52A1.

[0020] **SEQ ID NO. 9** is the nucleotide sequence of a gene from 69D1.

[0021] **SEQ ID NO. 10** is the amino acid sequence of the protein expressed by the gene from 69D1.

[0022] **SEQ ID NO. 11** is the nucleotide sequence of a gene from 63B.

[0023] **SEQ ID NO. 12** is the amino acid sequence of the protein expressed by the gene from 63B.

[0024] **SEQ ID NO. 13** is the amino acid sequence of a probe which can be used according to the subject invention.

[0025] **SEQ ID NO. 14** is the DNA coding for the amino acid sequence of SEQ ID NO. 13.

[0026] **SEQ ID NO. 15** is the amino acid sequence of a probe which can be used according to the subject invention.

[0027] **SEQ ID NO. 16** is the DNA coding for the amino acid sequence of SEQ ID NO. 15.

[0028] **SEQ ID NO. 17** is the N-terminal amino acid sequence of 17a.

[0029] **SEQ ID NO. 18** is the N-terminal amino acid sequence of 17b.

[0030] **SEQ ID NO. 19** is the N-terminal amino acid sequence of 52A1.

[0031] **SEQ ID NO. 20** is the N-terminal amino acid sequence of 63B.

[0032] **SEQ ID NO. 21** is the N-terminal amino acid sequence of 69D1.

[0033] **SEQ ID NO. 22** is the N-terminal amino acid sequence of 33F2.

[0034] **SEQ ID NO. 23** is an internal amino acid sequence for 63B.

[0035] **SEQ ID NO. 24** is a synthetic oligonucleotide derived from 17.

[0036] **SEQ ID NO. 25** is an oligonucleotide probe designed from the N-terminal amino acid sequence of 52A1.

[0037] **SEQ ID NO. 26** is the synthetic oligonucleotide probe designated as 69D1-D.

[0038] **SEQ ID NO. 27** is the forward oligonucleotide primer from 63B.

[0039] **SEQ ID NO. 28** is the reverse oligonucleotide primer from 63B.

[0040] **SEQ ID NO. 29** is the nematode (NEMI) variant of region 5 of Höfte and Whiteley.

[0041] **SEQ ID NO. 30** is the reverse complement primer to SEQ ID NO. 29, used according to the subject invention.

[0042] **SEQ ID NO. 31** is a peptide used according to the subject invention.

[0043] **SEQ ID NO. 32** is an oligonucleotide coding for the peptide of SEQ ID NO. 31.

[0044] **SEQ ID NO. 33** is oligonucleotide probe 33F2A.

[0045] **SEQ ID NO. 34** is oligonucleotide probe 33F2B.

[0046] **SEQ ID NO. 35** is a reverse primer used according to the subject invention.

[0047] **SEQ ID NO. 36** is a forward primer according to the subject invention.

[0048] **SEQ ID NO. 37** is a probe according to the subject invention.

[0049] **SEQ ID NO. 38** is a probe according to the subject invention.

[0050] **SEQ ID NO. 39** is a probe according to the subject invention.

[0051] **SEQ ID NO. 40** is a forward primer according to the subject invention.

Detailed Disclosure of the Invention

[0052] The subject invention concerns a vast array of *B.t.* δ-endotoxins having nematicidal activity. In addition to having nematicidal activity, the toxins of the subject invention will have one or more of the following characteristics:

1. An amino acid sequence according to either of the two generic formulae disclosed herein.
2. A high degree of amino acid homology with specific toxins disclosed herein.
3. A DNA sequence encoding the toxin which hybridizes with probes or genes disclosed herein.
4. A nucleotide sequence which can be amplified using primers disclosed herein.
5. A crystal toxin presentation as described herein.
6. Immunoreactivity to an antibody raised to a specific toxin disclosed herein.

[0053] One aspect of the subject invention concerns the discovery of generic chemical formulae which describe toxins having activity against nematodes. Two formulae are provided: one which pertains to nematicidal toxins having molecular weights of between about 45 kDa and 65 kDa, and the other pertains to larger nematicidal proteins having molecular weights from about 65 kDa to about 155 kDa. These formulae represent two different categories of *B.t.* δ-endotoxins, each of which has activity against nematodes. The formula describing smaller proteins describes many CryV proteins, while the formula describing larger proteins describes many CryVI proteins. A description of these two formulae is as follows:

[0054] Generic Formula I. This formula describes toxin proteins having molecular weights from about 65 kDa to about 155 kDa. The first 650-700 amino acids for proteins in excess of about 75 kDa and the entire molecule (for proteins of less than about 75 kDa) have substantially the following sequence:

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  1 MOXXXXXXPX BPYNBLOXXP XZXXXXXXXX OXxXXBXXXE UXBKXBJJXX
    XOxxxxZXXZ xXOBXJXBJX XBXXXXBXYX XXVUXZLZLB xxxXXOBPXB

101 ZBXXPBLZBB BXXBXXXXOx xxXUXOXLBX XBOXXBUJBL DJXLXXXXXX
    XLUXELXXBX XLXXKXXXXB XExxBXXHXX BXXBXXZXXX KBXXXXBZXX

201 ZBXOXXBXXB LOEXXXJxxx LXBPXYXBXO XMXLXXXXXX LXXZXOWXXK
    Bxxxxxxxxx XXXXOLXXXK XXBKXXLXBY XXXXXXBBXX XLXZXZxxZX

301 XXXBXJXXXY XJXMXXX*LE BXXXXPOBXP EXYxxxZZXL XLXKOKXLBZ
    XBBXXXXXxx XZBOLXUXXX XOXXXXXXXX ZXXXBXXXXJ JBXKxUBKBY

401 XXXXXXX*XX *Bx*YXXXBX BUXXXXOXXY ZXxxxXEPXX ZXXxxxBXXX
    XPBXXBUXXO XXOXXXXXXX XXOXXXKZXB *XLxxxxxxx *BXXKX*XXX

501 ZXZXZXZ*XX XLXZXXXXXX XXXXXXXXXX XZXXXxxxxx XLBXXXXPXE
    XXXXUXLZXX EXXZxUBXXX ZBPBEKxxOZ XXXXBxxBKE WLUZOXXXXL

601 ZPZUZXZBXB OUXOZZXYXB RCRYOZXXXO XBBBUxBXXZ ZXUPLXXUBX
    BXXOXEXXOX XXXXUXBXXB KZLXXXXXXB xxxxXxJLPX XXBXBXBOUX

701 ZSSXBXLDKL EBBPBX
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[0055] Numbering is for convenience and approximate location only.

Symbols used:

A = ala	G = gly	M = met	S = ser
C = cys	H = his	N = asn	T = thr
D = asp	I = ile	P = pro	V = val
E = glu	K = lys	Q = gln	W = trp
F = phe	L = leu	R = arg	Y =tyr

K = K or R	E = E or D	L = L or I
B = M, L, I, V, or F	J = K, R, E, or D	O = A or T
U = N or Q	Z = G or S	

X = any naturally occurring amino acid, except C.

* = any naturally occurring amino acid.

x = any naturally occurring amino acid, except C (or complete omission of any amino acids).

[0056] Where a stretch of wild-card amino acids are encountered (X(n) or x(n) where n>2), repetition of a given amino acid should be avoided. Similarly, P, C, E, D, K, or R utilization should be minimized.

[0057] This formula (hereinafter referred to as Generic Formula I) is exemplified in the current application by the specific toxins 17a, 17b and 63b.

[0058] Generic Formula II. This formula describes toxin proteins having molecular weights from about 45 kDa to about 65 kDa. Their primary amino acid structure substantially follows the motif illustrated below:

```
  1 MLBXXXXOBP KHxxxXXXXO XXXXZXKKxx xXZPXXBXXX XXBLLZKXEW
    OXBXOYBXOZ XZLPBUJXXB KXHBXLXXJL XLPXJBXULY JBYXXJKXXX

101 XWWUXXLXPL BBKXOUJLXX YZBKXOZJXX KKxxZXXJXB UJJBJULXJU
    XXJJOXXXKO XKJBXOKCXL LLKEOJUYJX OOJXBXXXLX XBLXZXUxxx

201 xXJBXZBXXB UXXLXXBXXX LXXXXZJXZP XXJELLJKBJ XLKXXLEXXL
    KOEUJLEKKB BXZBXLZPLL ZBBBYELLEX OOBXXLXXXB JXLXXXLJXO

301 UXJLJKJBKL LZBBUZLXOJ LJXBXXUZXX OLXBBXKLXZ LWXXLXXULX
    ULKXOZXXEB XJXXJXJXLX LELXJOXXXW XXBOXEOXXB XLUZYXXxxx

401 (x)n[a]
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[a]Where n = 0-100

[0059] The symbols used for this formula are the same as those used for Generic Formula I.

[0060] This formula (hereinafter referred to as Generic Formula II) is exemplified in the current application by specific toxins 52A1 and 69D1.

[0061] Nematode-active toxins according to the formulae of the subject invention are specifically exemplified herein by the toxins encoded by the genes designated 17a, 17b, 63B, 52A1, and 69D1. Since these toxins are merely exemplary of the toxins represented by the generic formulae presented herein, it should be readily apparent that the subject invention further comprises equivalent toxins (and nucleotide sequences coding for equivalent toxins) having the same or similar biological activity of the specific toxins disclosed or claimed herein. These equivalent toxins will have amino

acid homology with the toxins disclosed and claimed herein. This amino acid homology will typically be greater than 50%, preferably be greater than 75%, and most preferably be greater than 90%. The amino acid homology will be highest in certain critical regions of the toxin which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 1 provides a listing of examples of amino acids belonging to each class.

Table 1

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[0062] In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin. The information presented in the generic formulae of the subject invention provides clear guidance to the person skilled in this art in making various amino acid substitutions.

[0063] Further guidance for characterizing the nematicidal toxins of the subject invention is provided in Tables 3 and 4, which demonstrate the relatedness among toxins within each of the above-noted groups of nematicidal toxins (CryV and CryVI). These tables show a numeric score for the best matching alignment between two proteins that reflects: (1) positive scores for exact matches, (2) positive or negative scores reflecting the likelihood (or not) of one amino acid substituting for another in a related protein, and (3) negative scores for the introduction of gaps. A protein sequence aligned to itself will have the highest possible score—*i.e.*, all exact matches and no gaps. However, an unrelated protein or a randomly generated sequence will typically have a low

positive score. Related sequences have scores between the random background score and the perfect match score.

[0064] The sequence comparisons were made using the algorithm of Smith and Waterman ([1981] Advances in Applied Mathematics 2:482-489), implemented as the program "Bestfit" in the GCG Sequence Analysis Software Package Version 7 April 1991. The sequences were compared with default parameter values (comparison table: Swgappep.Cmp, Gap weight:3.0, Length weight:0.1) except that gap limits of 175 residues were applied to each sequence compared. The program output value compared is referred to as the Quality score.

[0065] Tables 3 and 4 show the pairwise alignments between the indicated amino acids of the two classes of nematode-active proteins CryV and CryVI and representatives of dipteran (CryIV; Sen, K. et al. [1988] Agric. Biol. Chem. 52:873-878), lepidopteran and dipteran (CryIIA; Widner and Whiteley [1989] J. Bacteriol. 171:965-974), lepidopteran (CryIA(c); Adang et al. [1981] Gene 36:289-300), and coleopteran (CryIIIA; Herrnstadt et al. [1987] Gene 57:37-46) proteins.

[0066] Table 2 shows which amino acids were compared from the proteins of interest.

Table 2

Protein	Amino acids compared
63B	1-692
33F2	1-618
17a	1-677
17b	1-678
CryIV	1-633
CryIIA	1-633
CryIA(c)	1-609
CryIIIA	1-644
69D1	1-395
52A1	1-475

Table 3 shows the scores prior to adjustment for random sequence scores.

Table 3

	63B	33F2	17a	CryIVA	CryIIA	CryIA(c)	CryIIIA	52A1	69D1
63B	1038	274	338	235	228	232	244	154	122
33F2		927	322	251	232	251	270	157	130
17a			1016	240	240	237	249	152	127
CryIVA				950	245	325	326	158	125
CryIIA					950	244	241	151	132
CryIA(c)						914	367	151	127
CryIIIA							966	150	123
52A1								713	350
69D1									593

[0067] Note that for each nematode-active protein, the highest score is always with another nematode-active protein. For example, 63B's highest score, aside from itself, is with 17a. Furthermore, 33F2's highest score, aside from itself, is also with 17a.

[0068] Similarly, 52A1 and 69D1 have a higher score versus each other than with the other proteins.

[0069] Table 4 shows the same analysis after subtraction of the average score of 50 alignments of random shuffles of the column sequences with the row sequences.

Table 4

	63B	33F2	17a	CryIVA	CryIIA	CryIA(c)	CryIIIA	52A1	69D1
63B	830	81	130	40	32	42	48	0.1	–8.8
33F2		740	128	66	48	72	85	1.4	–2.9
17a			808	45	45	45	54	–0.8	–5.2
CryIVA				759	54	142	138	5.4	–4.1
CryIIA					755	58	53	–2.3	6
CryIA(c)						728	185	3.1	0
CryIIIA							766	–2.3	–6.9
52A1								566	221
69D1									465

[0070] Note that in Table 4 the same relationships hold as in Table 3, i.e., 63B's highest score, aside from itself, is with 17a, and 33F2's highest score, aside from itself, is also with 17a.

[0071] Similarly, 52A1 and 69D1 have a better score versus each other than with the other proteins.

[0072] Thus, certain toxins according to the subject invention can be defined as those which have nematode activity and either have an alignment value (according to the procedures of Table 4) greater than 100 with 17a or have an alignment value greater than 100 with 52A1. As used herein,

the term "alignment value" refers to the scores obtained above and used to create the scores reported in Table 4.

[0073] The toxins of the subject invention can also be characterized in terms of the shape and location of crystal toxin inclusions. Specifically, nematode-active inclusions typically remain attached to the spore after cell lysis. These inclusions are not inside the exosporium, as in previous descriptions of attached inclusions, but are held within the spore by another mechanism. Inclusions of the nematode-active isolates are typically amorphic, generally long and/or multiple. These inclusions are distinguishable from the larger round/amorphic inclusions that remain attached to the spore. No *B.t.* strains that fit this description have been found to have activity against the conventional targets—Lepidoptera, Diptera, or Colorado Potato Beetle. All nematode-active strains fit this description except one. Thus, there is a very high correlation between this crystal structure and nematode activity.

[0074] The genes and toxins according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic nematicidal activity of the sequences specifically exemplified herein.

[0075] It should be apparent to a person skilled in this art that genes coding for nematode-active toxins can be identified and obtained through several means. The specific genes may be obtained from a culture depository as described below. These genes, or portions thereof, may be constructed synthetically, for example, by use of a gene machine. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of these toxins.

[0076] Equivalent toxins and/or genes encoding these equivalent toxins can also be located from *B.t.* isolates and/or DNA libraries using the teachings provided herein. There are a number of methods for obtaining the nematode-active toxins of the instant invention which occur in nature. For example, antibodies to the nematode-active toxins disclosed and claimed herein can be used to identify and isolate other toxins from a mixture of proteins. Specifically, antibodies may be raised to

the portions of the nematode-active toxins which are most constant and most distinct from other *B.t.* toxins. These antibodies can then be used to specifically identify equivalent toxins with the characteristic nematicidal activity by immunoprecipitation, enzyme linked immunoassay (ELISA), or Western blotting. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in this art. The genes coding for these toxins can then be obtained from the microorganism.

[0077] A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are nucleotide sequences having a detectable label. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample are essentially identical. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying nematicidal endotoxin genes of the subject invention.

[0078] The nucleotide segments which are used as probes according to the invention can be synthesized by use of DNA synthesizers using standard procedures. In the use of the nucleotide segments as probes, the particular probe is labeled with any suitable label known to those skilled in the art, including radioactive and non-radioactive labels. Typical radioactive labels include ^{32}P, ^{125}I, ^{35}S, or the like. A probe labeled with a radioactive isotope can be constructed from a nucleotide sequence complementary to the DNA sample by a conventional nick translation reaction, using a DNase and DNA polymerase. The probe and sample can then be combined in a hybridization buffer solution and held at an appropriate temperature until annealing occurs. Thereafter, the membrane is washed free of extraneous materials, leaving the sample and bound probe molecules typically detected and quantified by autoradiography and/or liquid scintillation counting.

[0079] Non-radioactive labels include, for example, ligands such as biotin or thyroxine, as well as enzymes such as hydrolases or perioxidases, or the various chemiluminescers such as luciferin, or fluorescent compounds like fluorescein and its derivatives. The probe may also be labeled at both ends with different types of labels for ease of separation, as, for example, by using an isotopic label at the end mentioned above and a biotin label at the other end.

[0080] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore,

the probes of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0081] The known methods include, but are not limited to:

(1) synthesizing chemically or otherwise an artificial sequence which is a mutation, insertion or deletion of the known sequence;

(2) using a probe of the present invention to obtain via hybridization a new sequence or a mutation, insertion or deletion of the probe sequence; and

(3) mutating, inserting or deleting a test sequence in vitro or in vivo.

[0082] It is important to note that the mutational, insertional, and deletional variants generated from a given probe may be more or less efficient than the original probe. Notwithstanding such differences in efficiency, these variants are within the scope of the present invention.

[0083] Thus, mutational, insertional, and deletional variants of the disclosed test sequences can be readily prepared by methods which are well known to those skilled in the art. These variants can be used in the same manner as the instant probes so long as the variants have substantial sequence homology with the probes. As used herein, substantial sequence homology refers to homology which is sufficient to enable the variant to function in the same capacity as the original probe. Preferably, this homology is greater than 50%; more preferably, this homology is greater than 75%; and most preferably, this homology is greater than 90%. The degree of homology needed for the variant to function in its intended capacity will depend upon the intended use of the sequence. It is well within the skill of a person trained in this art to make mutational, insertional, and deletional mutations which are designed to improve the function of the sequence or otherwise provide a methodological advantage.

[0084] Specific nucleotide probes useful, according to the subject invention, in the rapid identification of nematode-active genes are

(i) DNA coding for a peptide sequence whose single letter amino acid designation is "REWINGAN" (SEQ ID NO. 13) or variations thereof which embody point

mutations according to the following: position 1, R or P or K; position 3, W or Y; position 4, I or L; position 8, N or P; a specific example of such a probe is "AGA(A or G)T(G or A)(G or T)(A or T)T(A or T)AATGG(A or T)GC(G or T)(A or C)A(A or T)" (SEQ ID NO. 14);

(ii) DNA coding for a peptide sequence whose single letter amino acid designation is "PTFDPDLY" (SEQ ID NO. 15) or variations thereof which embody point mutations according to the following: position 3, F or L; position 4, D or Y; position 7, L or H or D; a specific example of such a probe is "CC(A or T)AC(C or T)TTT(T or G)ATCCAGAT(C or G)(T or A)TAT" (SEQ ID NO. 16).

[0085] The potential variations in the probes listed is due, in part, to the redundancy of the genetic code. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins. Therefore different nucleotide sequences can code for a particular amino acid. Thus, the amino acid sequences of the *B.t.* toxins and peptides can be prepared by equivalent nucleotide sequences encoding the same amino acid sequence of the protein or peptide. Accordingly, the subject invention includes such equivalent nucleotide sequences. Also, inverse or complement sequences are an aspect of the subject invention and can be readily used by a person skilled in this art. In addition it has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T. and Kezdy, F.J. [1984] Science 223:249-255). Thus, the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is substantially retained. Further, the invention also includes mutants of organisms hosting all or part of a toxin encoding a gene of the invention. Such microbial mutants can be made by techniques well known to persons skilled in the art. For example, UV irradiation can be used to prepare mutants of host organisms. Likewise, such mutants may include asporogenous host cells which also can be prepared by procedures well known in the art.

[0086] The toxin genes or gene fragments exemplified according to the subject invention can be obtained from nematode-active *B. thuringiensis* (*B.t.*) isolates designated PS17, PS33F2, PS63B, PS52A1, and PS69D1. Subcultures of the *E. coli* host harboring the toxin genes of the invention

were deposited in the permanent collection of the Northern Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, USA. The accession numbers are as follows:

Culture	Repository No.	Deposit Date
B.t. isolate PS17	NRRL B-18243	July 28, 1987
B.t. isolate PS33F2	NRRL B-18244	July 28, 1987
B.t. isolate PS63B	NRRL B-18246	July 28, 1987
B.t. isolate PS52A1	NRRL B-18245	July 28, 1987
B.t. isolate PS69D1	NRRL B-18247	July 28, 1987
E. coli NM522(pMYC 2316)	NRRL B-18785	March 15, 1991
E. coli NM522(pMYC 2321)	NRRL B-18770	February 14, 1991
E. coli NM522(pMYC 2317)	NRRL B-18816	April 24, 1991
E. coli NM522(pMYC 1627)	NRRL B-18651	May 11, 1990
E. coli NM522(pMYC 1628)	NRRL B-18652	May 11, 1990
E. coli NM522(pMYC 1642)	NRRL B-18961	April 10, 1992

[0087] The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

[0088] Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of

the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

[0089] The novel *B.t.* genes or gene fragments of the invention encode toxins which show activity against tested nematodes. The group of diseases described generally as helminthiasis is due to infection of an animal host with parasitic worms known as helminths. Helminthiasis is a prevalent and serious economic problem in domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. Among the helminths, the group of worms described as nematodes causes wide-spread and often times serious infection in various species of animals. The most common genera of nematodes infecting the animals referred to above are *Haemonchus, Trichostrongylus, Ostertagia, Nematodirus, Cooperia, Ascaris, Bunostomum, Oesophagostomum, Chabertia, Trichuris, Strongylus, Trichonema, Dictyocaulus, Capillaria, Heterakis, Toxocara, Ascaridia, Oxyuris, Ancylostoma, Uncinaria, Toxascaris, Caenorhabditis* and *Parascaris*. Certain of these, such as *Nematodirus, Cooperia*, and *Oesophagostomum*, attack primarily the intestinal tract, while others, such as *Dictyocaulus* are found in the lungs. Still other parasites may be located in other tissues and organs of the body.

[0090] The toxins encoded by the novel *B.t.* genes of the invention are useful as nematicides for the control of soil nematodes and plant parasites selected from the genera *Bursaphalenchus, Criconemella, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Melodoigyne, Pratylenchus, Radolpholus, Rotelynchus*, or *Tylenchus*.

[0091] Alternatively, because some plant parasitic nematodes are obligate parasites, genes coding for nematicidal *B.t.* toxins can be engineered into plant cells to yield nematode-resistant plants. The methodology for engineering plant cells is well established (cf. Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F., Ann. Rev. Plant Physiol. 35:387-399, 1984).

[0092] The *B.t.* toxins of the invention can be administered orally in a unit dosage form such as a capsule, bolus or tablet, or as a liquid drench when used as an anthelmintic in mammals, and in the soil to control plant nematodes. The drench is normally a solution, suspension or dispersion of the active ingredient, usually in water, together with a suspending agent such as bentonite and a wetting agent or like excipient. Generally, the drenches also contain an antifoaming agent. Drench formulations generally contain from about 0.001 to 0.5% by weight of the active compound. Preferred drench formulations may contain from 0.01 to 0.1% by weight, the capsules and boluses

comprise the active ingredient admixed with a carrier vehicle such as starch, talc, magnesium stearate, or dicalcium phosphate.

[0093] Where it is desired to administer the toxin compounds in a dry, solid unit dosage form, capsules, boluses or tablets containing the desired amount of active compound usually are employed. These dosage forms are prepared by intimately and uniformly mixing the active ingredient with suitable finely divided diluents, fillers, disintegrating agents and/or binders such as starch, lactose, talc, magnesium stearate, vegetable gums and the like. Such unit dosage formulations may be varied widely with respect to their total weight and content of the antiparasitic agent, depending upon the factors such as the type of host animal to be treated, the severity and type of infection and the weight of the host.

[0094] When the active compound is to be administered via an animal feedstuff, it is intimately dispersed in the feed or used as a top dressing or in the form of pellets which may then be added to the finished feed or, optionally, fed separately. Alternatively, the antiparasitic compounds may be administered to animals parenterally, for example, by intraruminal, intramuscular, intratracheal, or subcutaneous injection, in which event the active ingredient is dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material is suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety, such as peanut oil, cotton seed oil and the like. Other parenteral vehicles, such as organic preparations using solketal, glycerol, formal and aqueous parenteral formulations, are also used. The active compound or compounds are dissolved or suspended in the parenteral formulation for administration; such formulations generally contain from 0.005 to 5% by weight of the active compound.

[0095] When the toxins are administered as a component of the feed of the animals, or dissolved or suspended in the drinking water, compositions are provided in which the active compound or compounds are intimately dispersed in an inert carrier or diluent. By inert carrier is meant one that will not react with the antiparasitic agent and one that may be administered safely to animals. Preferably, a carrier for feed administration is one that is, or may be, an ingredient of the animal ration.

[0096] Suitable compositions include feed premixes or supplements in which the active ingredient is present in relatively large amounts and which are suitable for direct feeding to the animal or for addition to the feed either directly or after an intermediate dilution or blending step. Typical carriers

or diluents suitable for such compositions include, for example, distillers' dried grains, corn meal, citrus meal, fermentation residues, ground oyster shells, wheat shorts, molasses solubles, corn cob meal, edible bean mill feed, soya grits, crushed limestone and the like.

[0097] The toxin genes or gene fragments of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the nematicide. With suitable hosts, e.g., *Pseudomonas*, the microbes can be applied to the situs of nematodes where they will proliferate and be ingested by the nematodes. The result is a control of the nematodes. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the *B.t.* toxin.

[0098] Where the *B.t.* toxin gene or gene fragment is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the nematicide from environmental degradation and inactivation.

[0099] A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae. Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium*

melioti, Alcaligenes entrophus, and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae*, and *Aureobasidium pollulans*. Of particular interest are the pigmented microorganisms.

[00100] A wide variety of ways are known and available for introducing the *B.t.* genes or gene fragments expressing the toxin into the microorganism host under conditions which allow for stable maintenance and expression of the gene. The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for nematicidal activity.

[00101] Suitable host cells, where the nematicide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and -positive, include Enterobacteriaceae, such as *Escherichia, Erwinia, Shigella, Salmonella*, and *Proteus*; Bacillaceae; Rhizobiceae, such as *Rhizobium*; Spirillaceae, such as photobacterium, *Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum*; Lactobacillaceae; Pseudomonadaceae, such as *Pseudomonas* and *Acetobacter*; Azotobacteraceae and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as *Saccharomyces* and *Schizosaccharomyces*; and Basidiomycetes yeast, such as *Rhodotorula, Aureobasidium, Sporobolomyces*, and the like.

[00102] Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the *B.t.* gene or gene fragment into the host, availability of expression systems, efficiency of expression, stability of the nematicide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a nematicide microcapsule include protective qualities for the nematicide, such as thick cell walls, pigmentation, and intracellular packaging or

formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

[00103] Host organisms of particular interest include yeast, such as *Rhodotorula* sp., *Aureobasidium* sp., *Saccharomyces* sp., and *Sporobolomyces* sp.; phylloplane organisms such as *Pseudomonas* sp., *Erwinia* sp. and *Flavobacterium* sp.; or such other organisms as *Escherichia, Lactobacillus* sp., *Bacillus* sp., and the like. Specific organisms include *Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis*, and the like.

[00104] The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

[00105] Treatment of the microbial cell, e.g., a microbe containing the *B.t.* toxin gene or gene fragment, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

[00106] The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of inactivation should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

[00107] The cellular host containing the *B.t.* nematicidal gene or gene fragment may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B.t.* gene or gene fragment. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

[00108] The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. These procedures are all described in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York. Thus, it is within the skill of those in the genetic engineering art to extract DNA from microbial cells, perform restriction enzyme digestions, electrophorese DNA fragments, tail and anneal plasmid and insert DNA, ligate DNA, transform cells, prepare plasmid DNA, electrophorese proteins, and sequence DNA.

[00109] The *B.t.* cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

[00110] The nematicide concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The nematicide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the nematicide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

[00111] The formulations can be applied to the environment of the nematodes, e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

[00112] Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Culturing *B.t.* Isolates of the Invention

[00113] A subculture of a *B.t.* isolate can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.5 g/l
Glucose	1.0 g/l
KH_2PO_4	3.4 g/l
K_2HPO_4	4.35 g/l
Salts Solution	5.0 ml/l
$CaCl_2$ Solution	5.0 ml/l

Salts Solution (100 ml)

$MgSO_4.7H_2O$	2.46 g
$MnSO_4.H_2O$	0.04 g
$ZnSO_4.7H_2O$	0.28 g
$FeSO_4.7H_2O$	0.40 g

$CaCl_2$ Solution (100 ml)

$CaCl_2.2H_2O$	3.66 g
pH 7.2	

[00114] The salts solution and $CaCl_2$ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30°C on a rotary shaker at 200 rpm for 64 hr.

Example 2 – Purification of Protein and Amino Acid Sequencing

[00115] The *B.t.* isolates PS17, PS63B, PS52A1, and PS69D1 were cultured as described in Example 1. The parasporal inclusion bodies were partially purified by sodium bromide (28-38%) isopycnic gradient centrifugation (Pfannenstiel, M.A., E.J. Ross, V.C. Kramer, and K.W. Nickerson [1984]

FEMS Microbiol. Lett. 21:39). The proteins toxic for the nematode *Caenorhabditis elegans* were bound to PVDF membranes (Millipore, Bedford, MA) by western blotting techniques (Towbin, H., T. Staehlelin, and K. Gordon [1979] Proc. Natl. Acad. Sci. USA 76:4350) and the N-terminal amino acid sequences were determined by the standard Edman reaction with an automated gas-phase sequenator (Hunkapiller, M.W., R.M. Hewick, W.L. Dreyer, and L.E. Hood [1983] Meth. Enzymol. 91:399). The sequences obtained were:

PS17a: A I L N E L Y P S V P Y N V (SEQ ID NO. 17)

PS17b: A I L N E L Y P S V P Y N V (SEQ ID NO. 18)

PS52A1: M I I D S K T T L P R H S L I N T (SEQ ID NO. 19)

PS63B: Q L Q A Q P L I P Y N V L A (SEQ ID NO. 20)

PS69D1: M I L G N G K T L P K H I R L A H I F A T Q N S (SEQ ID NO. 21)

PS33F2: A T L N E V Y P V N (SEQ ID NO. 22)

[00116] In addition, internal amino acid sequence data were derived for PS63B. The toxin protein was partially digested with *Staphylococcus aureus* V8 protease (Sigma Chem. Co., St. Louis, MO) essentially as described (Cleveland, D.W., S.G. Fischer, M.W. Kirschner, and U.K. Laemmli [1977] J. Biol. Chem. 252:1102). The digested material was blotted onto PVDF membrane and a ca. 28 kDa limit peptide was selected for N-terminal sequencing as described above. The sequence obtained was:

PS63B(2) V Q R I L D E K L S F Q L I K (SEQ ID NO. 23)

[00117] From these sequence data oligonucleotide probes were designed by utilizing a codon frequency table assembled from available sequence data of other *B.t.* toxin genes. The probes were synthesized on an Applied Biosystems, Inc. DNA synthesis machine.

[00118] Protein purification and subsequent amino acid analysis of the N-terminal peptides listed above has led to the deduction of several oligonucleotide probes for the isolation of toxin genes from nematicidal *B.t.* isolates. RFLP analysis of restricted total cellular DNA using radiolabeled oligonucleotide probes has elucidated different genes or gene fragments.

Example 3 – Cloning of Novel Toxin Genes and Transformation into *Escherichia coli*

[00119] Total cellular DNA was prepared by growing the cells *B.t.* PS17 to a low optical density (OD_{600} = 1.0) and recovering the cells by centrifugation. The cells were protoplasted in TES buffer

(30 mM Tris-Cl, 10 mM EDTA, 50 mM NaCl, pH = 8.0) containing 20 % sucrose and 50 mg/ml lysozyme. The protoplasts were lysed by addition of SDS to a final concentration of 4%. The cellular material was precipitated overnight at 4°C in 100 mM (final concentration) neutral potassium chloride. The supernate was extracted twice with phenol/chloroform (1:1). The DNA was precipitated with ethanol and purified by isopycnic banding on a cesium chloride-ethidium bromide gradient.

[00120] Total cellular DNA from PS17 was digested with *Eco*RI and separated by electrophoresis on a 0.8% (w/v) Agarose-TAE (50 mM Tris-HCl, 20 mM NaOAc, 2.5 mM EDTA, pH=8.0) buffered gel. A Southern blot of the gel was hybridized with a [^{32}P] - radiolabeled oligonucleotide probe derived from the N-terminal amino acid sequence of purified 130 kDa protein from PS17. The sequence of the oligonucleotide synthesized is (GCAATTTTAAATGAATTATATCC) (SEQ ID NO. 24). Results showed that the hybridizing *Eco*RI fragments of PS17 are 5.0 kb, 4.5 kb, 2.7 kb and 1.8 kb in size, presumptively identifying at least four new nematode-active toxin genes, PS17d, PS17b, PS17a and PS17e, respectively.

[00121] A library was constructed from PS17 total cellular DNA partially digested with *Sau*3A and size fractionated by electrophoresis. The 9 to 23 kb region of the gel was excised and the DNA was electroeluted and then concentrated using an Elutip™ ion exchange column (Schleicher and Schuel, Keene NH). The isolated *Sau*3A fragments were ligated into LambdaGEM-11™ (PROMEGA). The packaged phage were plated on KW251 *E. coli* cells (PROMEGA) at a high titer and screened using the above radiolabeled synthetic oligonucleotide as a nucleic acid hybridization probe. Hybridizing plaques were purified and rescreened at a lower plaque density. Single isolated purified plaques that hybridized with the probe were used to infect KW251 *E. coli* cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures.

[00122] Recovered recombinant phage DNA was digested with *Eco*RI and separated by electrophoresis on a 0.8% agarose-TAE gel. The gel was Southern blotted and hybridized with the oligonucleotide probe to characterize the toxin genes isolated from the lambda library. Two patterns were present, clones containing the 4.5 kb (PS17b) or the 2.7 kb (PS17a) *Eco*RI fragments. Preparative amounts of phage DNA were digested with *Sal*I (to release the inserted DNA from lambda arms) and separated by electrophoresis on a 0.6% agarose-TAE gel. The large fragments, electroeluted and concentrated as described above, were ligated to *Sal*I-digested and

dephosphorylated pBClac, an *E. coli/B.t.* shuttle vector comprised of replication origins from pBC16 and pUC19 . The ligation mix was introduced by transformation into NM522 competent *E. coli* cells and plated on LB agar containing ampicillin, isopropyl-(Beta)-D-thiogalactoside (IPTG) and 5-Bromo-4-Chloro-3-indolyl-(Beta)-D-galactoside (XGAL). White colonies, with putative insertions in the (Beta)-galactosidase gene of pBClac, were subjected to standard rapid plasmid purification procedures to isolate the desired plasmids. The selected plasmid containing the 2.7 kb *Eco*RI fragment was named pMYC1627 and the plasmid containing the 4.5 kb *Eco*RI fragment was called pMYC1628.

[00123] The toxin genes were sequenced by the standard Sanger dideoxy chain termination method using the synthetic oligonucleotide probe, disclosed above, and by "walking" with primers made to the sequence of the new toxin genes.

[00124] The PS17 toxin genes were subcloned into the shuttle vector pHT3101 (Lereclus, D. et al. [1989] FEMS Microbiol. Lett. 60:211-218) using standard methods for expression in *B.t.* Briefly, *Sal*I fragments containing the 17a and 17b toxin genes were isolated from pMYC1629 and pMYC1627, respectively, by preparative agarose gel electrophoresis, electroelution, and concentrated, as described above. These concentrated fragments were ligated into *Sal*I-cleaved and dephosphorylated pHT3101. The ligation mixtures were used separately to transform frozen, competent *E. coli* NM522. Plasmids from each respective recombinant *E. coli* strain were prepared by alkaline lysis and analyzed by agarose gel electrophoresis. The resulting subclones, pMYC2311 and pMYC2309, harbored the 17a and 17b toxin genes, respectively. These plasmids were transformed into the acrystalliferous *B.t.* strain, HD-1 *cry*B (Aronson, A., Purdue University, West Lafayette, IN), by standard electroporation techniques (Instruction Manual, Biorad, Richmond, CA).

[00125] Recombinant *B.t.* strains HD-1 *cry*B [pMYC2311] and [pMYC2309] were grown to sporulation and the proteins purified by NaBr gradient centrifugation as described above for the wild-type *B.t.* proteins.

Example 4 – Activity of the *B.t.* Toxin Protein and Gene Product Against *Caenorhabditis elegans*

[00126] *Caenorhabditis elegans* (CE) was cultured as described by Simpkin and Coles (J. Chem. Tech. Biotechnol. 31:66-69, 1981) in corning (Corning Glass Works, Corning, NY) 24-well tissue culture plates containing 1 ml S-basal media, 0.5 mg ampicillin and 0.01 mg cholesterol. Each well

also contained *ca.* 10^8 cells of *Escherichia coli* strain OP-50, a uracil auxotroph. The wells were seeded with *ca.* 100-200 CE per well and incubated at 20°C. Samples of protein (obtained from the wild type *B.t.* or the recombinant *B.t.*) were added to the wells by serial dilution. Water served as the control as well as the vehicle to introduce the proteins to the wells.

[00127] Each of the wells were examined daily and representative results are shown in Table 5 as follows:

Table 5

	% Kill with protein from indicated isolate		
μg Toxin	HD-1 cryB [pMYC2309]	HD-1 cryB [pMYC 2311]	PS17
100	25	50	75
32	25	50	75
10	50	25	50
1	0	0	0

Example 5 – Molecular Cloning of Gene Encoding a Novel Toxin From *Bacillus thuringiensis* strain PS52A1

[00128] Total cellular DNA was prepared from *Bacillus thuringiensis* PS52A1 (B.t. PS52A1) as disclosed in Example 3.

[00129] RFLP analyses were performed by standard hybridization of Southern blots of PS52A1 DNA with a ^{32}P-labeled oligonucleotide probe designed from the N-terminal amino acid sequence disclosed in Example 2. The sequence of this probe is:

5' ATG ATT ATT GAT TCT AAA ACA ACA TTA CCA AGA CAT TCA/T TTA ATA/T AAT ACA/T ATA/T AA 3' (SEQ ID NO. 25)

This probe was designated 52A1-C. Hybridizing bands included an approximately 3.6 kbp *Hin*dIII fragment and an approximately 8.6 kbp *Eco*RV fragment. A gene library was constructed from PS52A1 DNA partially digested with *Sau*3A. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 6.6 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, and recovered by ethanol precipitation after purification on an Elutip-D ion exchange column. The *Sau*3A inserts were ligated into *Bam*HI-digested LambdaGem-11 (Promega). Recombinant phage were packaged and plated on *E. coli* KW251 cells (Promega). Plaques were screened by hybridization with the radiolabeled 52A1-C oligonucleotide probe disclosed above. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for

isolation of phage DNA by standard procedures (Maniatis et al.). For subcloning, preparative amounts of DNA were digested with *Eco*RI and *Sal*I, and electrophoresed on an agarose gel. The approximately 3.1 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as above. The purified DNA insert was ligated into *Eco*RI + *Sal*I-digested pHTBlueII (an *E. coli/B. thuringiensis* shuttle vector comprised of pBluescript S/K [Stratagene] and the replication origin from a resident *B.t.* plasmid [D. Lereclus et al. 1989. FEMS Microbiology Letters 60:211-218]). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). Transformants were plated on LB agar containing ampicillin, isopropyl-(Beta)-D-thiogalactoside (IPTG), and 5-Bromo-4-Chloro-3-indolyl-(Beta)-D-galactoside (XGAL). Plasmids were purified from putative recombinants by alkaline lysis (Maniatis et al.) and analyzed by electrophoresis of *Eco*RI and *Sal*I digests on agarose gels. The desired plasmid construct, pMYC2321 contains a toxin gene that is novel compared to the maps of other toxin genes encoding nematicidal proteins.

[00130] Plasmid pMYC2321 was introduced into an acrystalliferous (Cry^-) *B.t.* host by electroporation. Expression of an approximately 55-60 kDa crystal protein was verified by SDS-PAGE analysis. NaBr-purified crystals were prepared as described in Example 3 for determination of toxicity of the cloned gene product to *Pratylenchus* spp.

Example 6 – Activity of the *B.t.* PS52A1 Toxin Protein and Gene Product Against the Root Lesion Nematode, *Pratylenchus scribneri*

[00131] *Pratylenchus scribneri* was reared aseptically on excised corn roots in Gamborg's B5 medium (GIBCO Laboratories, Grand Island, NY). Bioassays were done in 24 well assay plates (Corning #25820) using L 3-4 larvae as described by Tsai and Van Gundy (J. Nematol. 22(3):327-332). Approximately 20 nematodes were placed in each well. A total of 80-160 nematodes were used in each treatment. Samples of protein were suspended in aqueous solution using a hand-held homogenizer.

[00132] Mortality was assessed by prodding with a dull probe 7 days after treatment. Larvae that did not respond to prodding were considered moribund. Representative results are shown below.

Rate (ppm)	Percent Moribund
200	75
Control	5

Example 7 – Molecular Cloning of Gene Encoding a Novel Toxin From *Bacillus Thuringiensis* strain PS69D1

[00133] Total cellular DNA was prepared from PS69D1 (*B.t.* PS69D1) as disclosed in Example 3. RFLP analyses were performed by standard hybridization of Southern blots of PS69D1 DNA with a 32P-labeled oligonucleotide probe designated as 69D1-D. The sequence of the 69D1-D probe was:

[00134] 5' AAA CAT ATT AGA TTA GCA CAT ATT TTT GCA ACA CAA AA 3' (SEQ ID NO. 26)

[00135] Hybridizing bands included an approximately 2.0 kbp *Hin*dIII fragment.

[00136] A gene library was constructed from PS69D1 DNA partially digested with *Sau*3A. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 6.6 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, and recovered by ethanol precipitation after purification on an Elutip-D ion exchange column. The *Sau*3A inserts were ligated into *Bam*HI-digested LambdaGem-11 (Promega, Madison, WI). Recombinant phage were packaged and plated on *E. coli* KW251 cells (Promega, Madison, WI). Plaques were screened by hybridization with the radiolabeled 69D1-D oligonucleotide probe. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for isolation of phage DNA by standard procedures (Maniatis et al. [1982] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY). For subcloning, preparative amounts of DNA were digested with *Hin*dIII and electrophoresed on an agarose gel. The approximately, 2.0 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as above. The purified DNA insert was ligated into *Hin*dIII-digested pHTBlueII (and *E. coli/B.t.* shuttle vector comprised of pBluescript S/K (Stratagene, San Diego, CA) and the replication origin from a resident *B.t.* plasmid (D. Lereclus et al [1989] FEMS Microbiol. Lett. 60:211-218). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). Transformants were plated on LB agar containing 5-bromo-4-chloro-3-indolyl-(Beta)-D-galactoside (XGAL). Plasmids were purified from putative recombinants by alkaline lysis (Maniatis et al., *supra*) and analyzed by electrophoresis of *Hin*dIII digests on agarose gels. The desired plasmid

construct, pMYC2317, contains a toxin gene that is novel compared to the maps of other toxin genes encoding insecticidal proteins.

Example 8 – Molecular Cloning of a Gene Encoding a Novel Toxin from *Bacillus thuringiensis* Strain PS63B

[00137] Example 2 shows the aminoterminal and internal polypeptide sequences of the PS63B toxin protein as determined by standard Edman protein sequencing. From these sequences, two oligonucleotide primers were designed using a codon frequency table assembled from B.t. genes encoding δ-endotoxins. The sequence of the forward primer (63B-A) was complementary to the predicted DNA sequence at the 5' end of the gene:

63B-A - 5' CAA T/CTA CAA GCA/T CAA CC 3' (SEQ ID NO. 27)

The sequence of the reverse primer (63B-INT) was complementary to the inverse of the internal predicted DNA sequence:

63B-INT - 5' TTC ATC TAA AAT TCT TTG A/TAC 3' (SEQ ID NO. 28)

These primers were used in standard polymerase chain reactions (Cetus Corporation) to amplify an approximately 460 bp fragment of the 63B toxin gene for use as a DNA cloning probe. Standard Southern blots of total cellular DNA from PS63B were hybridized with the radiolabeled PCR probe. Hybridizing bands included an approximately 4.4 kbp *Xba*I fragment, an approximately 2.0 kbp *Hin*dIII fragment, and an approximately 6.4 kbp *Spe*I fragment.

[00138] Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density of 1.0 at 600 nm. The cells were recovered by centrifugation and protoplasts were prepared in lysis mix (300 mM sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH = 8.0) and lysozyme at a concentration of 20 mg/ml. The protoplasts were ruptured by addition of ten volumes of 0.1 M NaCl, 0.1 M Tris-HCl pH 8.0, and 0.1% SDS. The cellular material was quickly frozen at –70°C and thawed to 37°C twice. The supernatant was extracted twice with phenol/chloroform (1:1). The nucleic acids were precipitated with ethanol. To remove as much RNA as possible from the DNA preparation, RNase at final concentration of 200 μg/ml was added. After incubation at 37°C for 1 hour, the solution was extracted once with phenol/chloroform and precipitated with ethanol.

[00139] A gene library was constructed from PS63B total cellular DNA partially digested with *Nde*II and size fractioned by gel electrophoresis. The 9-23 kb region of the gel was excised and the DNA

was electroeluted and then concentrated using an Elutip-d ion exchange column (Schleicher and Schuel, Keene, NH). The isolated *Nde*II fragments were ligated into *Bam*HI-digested LambdaGEM-11 (PROMEGA). The packaged phage were plated on *E. coli* KW251 cells (PROMEGA) at a high titer and screened using the radiolabeled approximately 430 bp fragment probe amplified with the 63B-A and 63B internal primers (SEQ ID NOS. 27 and 28, respectively) by polymerase chain reaction. Hybridizing plaques were purified and rescreened at a lower plaque density. Single isolated, purified plaques that hybridized with the probe were used to infect KW251 cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures (Maniatis et al., *supra*). Preparative amounts of DNA were digested with *Sal*I (to release the inserted DNA from lambda sequences) and separated by electrophoresis on a 0.6% agarose-TAE gel. The large fragments were purified by ion exchange chromatography as above and ligated to *Sal*I-digested, dephosphorylated pHTBlueII (an *E. coli/B.t.* shuttle vector comprised of pBlueScript S/K [Stratagene, San Diego, CA] and the replication origin from a resident *B.t.* plasmid [Lereclus, D. et al. (1989) FEMS Microbiol. Lett. 60:211-218]). The ligation mix was introduced by transformation into competent *E. coli* NM522 cells (ATCC 47000) and plated on LB agar containing ampicillin (100 μg/ml), IPTG (2%), and XGAL (2%). White colonies, with putative restriction fragment insertions in the (Beta)-galactosidase gene of pHTBlueII, were subjected to standard rapid plasmid purification procedures (Maniatis et al., *supra*). Plasmids ere analyzed by *Sal*I digestion and agarose gel electrophoresis. The desired plasmid construct, pMYC1641, contains an approximately 14 kb *Sal*I insert.

[00140] For subcloning, preparative amounts of DNA were digested with *Xba*I and electrophoresed on an agarose gel. The approximately 4.4 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as above. This fragment was ligated into *Xba*I cut pHTBlueII and the resultant plasmid was designated pMYC1642.

Example 9 – Cloning of a Novel Toxin Gene From *B.t.* PS33F2 and Transformation into *Escherichia coli*

[00141] Total cellular DNA was prepared from *B.t.* PS33F2 cells grown to an optical density, at 600 nm, of 1.0. Cells were pelleted by centrifugation and resuspended in protoplast buffer (20 mg/ml lysozyme in 0.3 M sucrose, 25 mM Tris-Cl [pH 8.0], 25 mM EDTA). After incubation at 37°C for 1

hour, protoplasts were lysed by the addition of nine volumes of a solution of 0.1 M NaCl, 0.1% SDS, 0.1 M Tris-Cl followed by two cycles of freezing and thawing. The cleared lysate was extracted twice with phenol:chloroform (1:1). Nucleic acids were precipitated with two volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA (TE) and RNase was added to a final concentration of 50 μg/ml. After incubation at 37°C for 1 hour, the solution was extracted once each with phenol:chloroform (1:1) and TE-saturated chloroform. DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M NaOAc and two volumes of ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in TE.

[00142] Plasmid DNA was extracted from protoplasts prepared as described above. Protoplasts were lysed by the addition of nine volumes of a solution of 10 mM Tris-Cl, 1 mM EDTA, 0.085 N NaOH, 0.1% SDS, pH=8.0. SDS was added to 1% final concentration to complete lysis. One-half volume of 3 M KOAc was then added and the cellular material was precipitated overnight at 4°C. After centrifugation, the DNA was precipitated with ethanol and plasmids were purified by isopycnic centrifugation on cesium chloride-ethidium bromide gradients.

[00143] Restriction Fragment Length Polymorphism (RFLP) analyses were performed by standard hybridization of Southern blots of PS33F2 plasmid and total cellular DNA with the following two ^{32}P-labelled oligonucleotide probes (designed to the N-terminal amino acid sequence disclosed in Example 2):

Probe 33F2A: 5′ GCA/T ACA/T TTA AAT GAA GTA/T TAT 3′ **(SEQ ID NO. 33)**

Probe 33F2B: 5′ AAT GAA GTA/T TAT CCA/T GTA/T AAT 3′ **(SEQ ID NO. 34)**

Hybridizing bands included an approximately 5.85 kbp *Eco*RI fragment. Probe 33F2A and a reverse PCR primer were used to amplify a DNA fragment of approximately 1.8 kbp for use as a hybridization probe for cloning the PS33F2 toxin gene. The sequence of the reverse primer was:

5′ GCAAGCGGCCGCTTATGGAATAAATTCAATT C/T T/G A/G TC T/A A 3′ **(SEQ ID NO. 35)**.

[00144] A gene library was constructed from PS33F2 plasmid DNA digested with *Eco*RI. Restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 4.3-6.6 kbp were excised from the gel, electroeluted from the gel slice, and recovered by ethanol precipitation after purification on an Elutip-D ion exchange column (Schleicher and Schuel, Keene NH). The *Eco*RI

inserts were ligated into *Eco*RI-digested pHTBlueII (an *E. coli/B. thuringiensis* shuttle vector comprised of pBluescript S/K [Stratagene] and the replication origin from a resident *B.t.* plasmid [D. Lereclus et al. 1989. FEMS Microbial. Lett. 60:211-218]). The ligation mixture was transformed into frozen, competent NM522 cells (ATCC 47000). Transformants were plated on LB agar containing ampicillin, isopropyl -(Beta)-D-thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-(Beta)-D-galactoside (XGAL). Colonies were screened by hybridization with the radiolabeled PCR amplified probe described above. Plasmids were purified from putative toxin gene clones by alkaline lysis and analyzed by agarose gel electrophoresis of restriction digests. The desired plasmid construct, pMYC2316, contains an approximately 5.85 kbp *Eco*4RI insert; the toxin gene residing on this DNA fragment (33F2a) is novel compared to the DNA sequences of other toxin genes encoding nematicidal proteins.

[00145] Plasmid pMYC2316 was introduced into the acrystalliferous (Cry-) *B.t.* host, HD-1 CryB (A. Aronson, Purdue University, West Lafayette, IN) by electroporation. Expression of an approximately 120-140 kDa crystal protein was verified by SDS-PAGE analysis. Crystals were purified on NaBr gradients (M.A. Pfannenstiel et al. 1984. FEMS Microbiol. Lett. 21:39) for determination of toxicity of the cloned gene product to *Pratylenchus* spp.

Example 10 – Activity of the *B.t.* Gene Product 33F2 Against the Plant Nematode *Pratylenchus* spp.

[00146] *Pratylenchus* spp. was reared aseptically on excised corn roots in Gamborg's B5 medium (GIBCO® Laboratories, Grand Island, NY) Bioassays were done in 24 well assay plates (Corning #25820) using L 3-4 larvae as described by Tsai and van Gundy (J. Nematol. 22(3):327-332). Approximately 20 nematodes were placed in each well. A total of 80-160 nematodes were used in each treatment. Samples of protein were suspended in an aqueous solution using a hand-held Dounce homogenizer.

[00147] Mortality was assessed visually 3 days after treatment. Larvae that were nearly straight and not moving were considered moribund. Representative results are as follows:

33F2a (ppm)	% Moribund
0	12
75	78

[00148] Species of *Pratylenchus*, for example *P. scribneri*, are known pathogens of many economically important crops including corn, peanuts, soybean, alfalfa, beans, tomato, and citrus. These "root lesion" nematodes are the second most economically damaging genus of plant parasitic nematodes (after *Meloidogyne*—the "root knot" nematode), and typify the migratory endoparasites.

Example 11 – Cloning of Novel Nematode-Active Genes Using Generic Oligonucleotide Primers

[00149] The nematicidal gene of a new nematicidal *B.t.* can be obtained from DNA of the strain by performing the standard polymerase chain reaction procedure as in Example 8 using the oligonucleotides of SEQ ID NO. 32 or SEQ ID NO. 30 as reverse primers and SEQ ID NO. 14, SEQ ID NO. 16, SEQ ID NO. 24, Probe B of SEQ ID NO. 5 (AAT GAA GTA/T TAT CCA/T GTA/T AAT), or SEQ ID NO. 27 as forward primers. The expected PCR fragments would be approximately 330 to 600 bp (with either reverse primer and SEQ ID NO. 14), 1000 to 1400 bp (with either reverse primer and SEQ ID NO. 16), and 1800 to 2100 bp (with either reverse primer and any of the three N-terminal primers, SEQ ID NO. 5 (Probe B), SEQ ID NO. 24, and SEQ ID NO. 27). Alternatively, a complement from the primer family described by SEQ ID NO. 14 can be used as reverse primer with SEQ ID NO. 16, SEQ ID NO. 24, SEQ ID NO. 5 (Probe B), or SEQ ID NO. 27 as forward primers. The expected PCR fragments would be approximately 650 to 1000 bp with SEQ ID NO. 16, and 1400 to 1800 bp (for the three N-terminal primers, SEQ ID NO. 5 (Probe B), SEQ ID NO. 24, and SEQ ID NO. 27). Amplified DNA fragments of the indicated sizes can be radiolabeled and used as probes to clone the entire gene as in Example 8.

Example 12 – Further Cloning of Novel Nematode-Active Genes Using Generic Oligonucleotide Primers

[00150] A gene coding for a nematicidal toxin a new nematicidal *B.t.* isolate can also be obtained from DNA of the strain by performing the standard polymerase chain reaction procedure as in Example 8 using oligonucleotides derived from the PS52A1 and PS69D1 gene sequences as follows:

1. Forward primer "TGATTTT(T or A)(C or A)TCAATTATAT(A or G)A(G or T)GTTTAT" (SEQ ID NO. 36) can be used with primers complementary to probe "AAGAGTTA(C or T)TA(A or G)A(G or A)AAAGTA" (SEQ ID NO. 37), probe "TTAGGACCATT(A or G)(C or

T)T(T or A)GGATTTGTTGT(A or T)TATGAAAT" (SEQ ID NO. 38), and probe "GA(C or T)AGAGATGT(A or T)AAAAT(C or T)(T or A)TAGGAATG" (SEQ ID NO. 39) to produce amplified fragments of approximately 440, 540, and 650 bp, respectively.

2. Forward primer "TT(A or C)TTAAA(A or T)C(A or T)GCTAATGATATT" (SEQ ID NO. 40) can be used with primers complementary to SEQ ID NO. 37, SEQ ID NO. 38, and SEQ ID NO. 39 to produce amplified fragments of approximately 360, 460, and 570 bp, respectively.

3. Forward primer SEQ ID NO. 37 can be used with primers complementary to SEQ ID NO. 38 and SEQ ID NO. 39 to produce amplified fragments of approximately 100 and 215 bp, respectively.

[00151] Amplified DNA fragments of the indicated sizes can be radiolabeled and used as probes to clone the entire gene as in Example 8.

Example 13 – Insertion of Toxin Gene Into Plants

[00152] One aspect of the subject invention is the transformation of plants with genes coding for a nematicidal toxin. The transformed plants are resistant to attack by nematodes.

[00153] Genes coding for nematicidal toxins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence coding for the *B.t.* toxin can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

[00154] The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasserdam, Chapter 5; Fraley *et al.*, Crit. Rev. Plant Sci. 4:1-46; and An *et al.* (1985) EMBO J. 4:277-287.

[00155] Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

[00156] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, or electroporation as well as other possible methods. If agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.* [1978] Mol. Gen. Genet. 163:181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be

tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00157] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00158] It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.